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Under Secretary of Commerce
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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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☐ *Additional inventors are being named on the separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

PEG-YLATED NUCLEOTIDE SUGARS

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages* 24 ☐ CD(s), Number

☒ Drawing(s) *Number of Sheets* 2 ☐ Other (specify)

☒ Application Data Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☒ Applicant claims small entity status. See 37 CFR 1.27.

☐ A check or money order is enclosed to cover the filing fees

FILING FEE Amount ($)

☒ The Director is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: **20-1430** — 80

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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are: .

Respectfully submitted,

SIGNATURE

Date 01/26/04

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USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

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PROVISIONAL

PATENT APPLICATION

PEG-YLATED NUCLEOTIDE SUGARS

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AS FILED IN THE USPTO ON JANUARY 26, 2004

PEG-YLATED NUCLEOTIDE SUGARS

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] NOT APPLICABLE

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

[0002] NOT APPLICABLE

REFERENCE TO A "SEQUENCE LISTING," A TABLE, OR A COMPUTER PROGRAM LISTING APPENDIX SUBMITTED ON A COMPACT DISK.

[0003] NOT APPLICABLE

BACKGROUND OF THE INVENTION

[0004] Post-expression *in vitro* modification of peptides is an attractive strategy to remedy the deficiencies of methods that rely on controlling glycosylation by engineering expression systems; including both modification of glycan structures or introduction of glycans at novel sites. A comprehensive toolbox of recombinant eukaryotic glycosyltransferases is becoming available, making *in vitro* enzymatic synthesis of mammalian glycoconjugates with custom designed glycosylation patterns and glycosyl structures possible. See, for example, U.S. Patent No. 5,876,980; 6,030,815; 5,728,554; 5,922,577; and WO/9831826; US2003180835; and WO 03/031464.

[0005] Enzyme-based syntheses have the advantages of regioselectivity and stereoselectivity. Moreover, enzymatic syntheses are performed using unprotected substrates. Three principal classes of enzymes are used in the synthesis of carbohydrates, glycosyltransferases (*e.g.*, sialyltransferases, oligosaccharyltransferases, N-acetylglucosaminyltransferases), and glycosidases. The glycosidases are further classified as exoglycosidases (*e.g.*, β-mannosidase, β-glucosidase), and endoglycosidases (*e.g.*, Endo-A, Endo-M). Each of these classes of enzymes has been successfully used synthetically to

prepare carbohydrates. For a general review, *see,* Crout *et al., Curr. Opin. Chem. Biol.* **2**: 98-111 (1998).

[0006] Glycosyltransferases modify the oligosaccharide structures on glycopeptides. Glycosyltransferases are effective for producing specific products with good stereochemical and regiochemical control. Glycosyltransferases have been used to prepare oligosaccharides and to modify terminal N- and O-linked carbohydrate structures, particularly on glycopeptides produced in mammalian cells. For example, the terminal oligosaccharides of glycopeptides have been completely sialylated and/or fucosylated to provide more consistent sugar structures, which improves glycopeptide pharmacodynamics and a variety of other biological properties. For example, β-1,4-galactosyltransferase was used to synthesize lactosamine, an illustration of the utility of glycosyltransferases in the synthesis of carbohydrates (*see, e.g.*, Wong *et al., J. Org. Chem.* **47**: 5416-5418 (1982)). Moreover, numerous synthetic procedures have made use of α-sialyltransferases to transfer sialic acid from cytidine-5'-monophospho-N-acetylneuraminic acid to the 3-OH or 6-OH of galactose (*see, e.g.*, Kevin *et al., Chem. Eur. J.* **2**: 1359-1362 (1996)). Fucosyltransferases are used in synthetic pathways to transfer a fucose unit from guanosine-5'-diphosphofucose to a specific hydroxyl of a saccharide acceptor. For example, Ichikawa prepared sialyl Lewis-X by a method that involves the fucosylation of sialylated lactosamine with a cloned fucosyltransferase (Ichikawa *et al., J. Am. Chem. Soc.* **114**: 9283-9298 (1992)). For a discussion of recent advances in glycoconjugate synthesis for therapeutic use *see,* Koeller *et al., Nature Biotechnology* **18**: 835-841 (2000). *See* also, U.S. Patent No. 5,876,980; 6,030,815; 5,728,554; 5,922,577; and WO/9831826.

[0007] In addition to manipulating the structure of glycosyl groups on polypeptides, interest has developed in preparing glycopeptides that are modified with one or more non-saccharide modifying group, such as water soluble polymers. Poly(ethyleneglycol) ("PEG") is an exemplary polymer that has been conjugated to polypeptides. The use of PEG to derivatize peptide therapeutics has been demonstrated to reduce the immunogenicity of the peptides. For example, U.S. Pat. No. 4,179,337 (Davis *et al.*) discloses non-immunogenic polypeptides, such as enzymes and peptide hormones coupled to polyethylene glycol (PEG) or polypropylene glycol. Between 10 and 100 moles of polymer are used per mole polypeptide. Although the in vivo clearance time of the conjugate is prolonged relative to that of the polypeptide, only about 15% of the physiological activity is maintained. Thus, the

prolonged circulation half-life is counterbalanced by the dramatic reduction in peptide potency.

[0008] The loss of peptide activity is directly attributable to the non-selective nature of the chemistries utilized to conjugate the water-soluble polymer. The principal mode of attachment of PEG, and its derivatives, to peptides is a non-specific bonding through a peptide amino acid residue. For example, U.S. Patent No. 4,088,538 discloses an enzymatically active polymer-enzyme conjugate of an enzyme covalently bound to PEG. Similarly, U.S. Patent No. 4,496,689 discloses a covalently attached complex of α-1 proteinase inhibitor with a polymer such as PEG or methoxypoly(ethyleneglycol) ("MPEG"). Abuchowski *et al.* (*J. Biol. Chem.* **252**: 3578 (1977) discloses the covalent attachment of MPEG to an amine group of bovine serum albumin. U.S. Patent No. 4,414,147 discloses a method of rendering interferon less hydrophobic by conjugating it to an anhydride of a dicarboxylic acid, such as poly(ethylene succinic anhydride). PCT WO 87/00056 discloses conjugation of PEG and poly(oxyethylated) polyols to such proteins as interferon-β, interleukin-2 and immunotoxins. EP 154,316 discloses and claims chemically modified lymphokines, such as IL-2 containing PEG bonded directly to at least one primary amino group of the lymphokine. U.S. Patent No. 4,055,635 discloses pharmaceutical compositions of a water-soluble complex of a proteolytic enzyme linked covalently to a polymeric substance such as a polysaccharide.

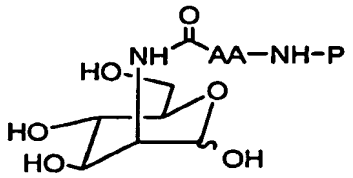
[0009] Another mode of attaching PEG to peptides is through the non-specific oxidation of glycosyl residues on a glycopeptide. The oxidized sugar is utilized as a locus for attaching a PEG moiety to the peptide. For example M'Timkulu (WO 94/05332) discloses the use of an amino-PEG to add PEG to a glycoprotein. The glycosyl moieties are randomly oxidized to the corresponding aldehydes, which are subsequently coupled to the amino-PEG.

[0010] In each of the methods described above, poly(ethyleneglycol) is added in a random, non-specific manner to reactive residues on a peptide backbone. For the production of therapeutic peptides, it is clearly desirable to utilize a derivitization strategy that results in the formation of a specifically labeled, readily characterizable, essentially homogeneous product. A promising route to preparing specifically labeled peptides is through the use of enzymes, such as glycosyltransferases to append a modified sugar moiety onto a peptide. The modified sugar moiety must function as a substrate for the glycosyltransferase and be appropriately

activated. Hence, synthetic routes that provide facile access to activated modified sugars are desirable. The present invention provides such a route.

BRIEF SUMMARY OF THE INVENTION

[0011] It has now been discovered that a modified sialic acid that is activated as a nucleotide sugar is readily prepared using a synthetic scheme that proceeds through an amide conjugate formed between mannosamine and a protected amino acid. An exemplary conjugate has the formula:



in which AA-NH is that portion of an amino acid residue that does not include the carboxyl moiety and P is a protecting group.

[0012] The conjugate is readily converted to a modified, activated sialic acid species that is of use in the glycoconjugation of the modifying moiety to another species, e.g., peptide, lipid, nucleic acid, etc. through an intact glycosyl linking group.

[0013] Also provided is a synthetic method for producing an activated sialic acid-PEG conjugate that is an appropriate substrate for a glycosyltransferase. The method utilizes the above-described conjugate. The method includes the steps: (a) contacting mannosamine with an activated, N-protected amino acid under conditions appropriate to form an amide conjugate between the mannosamine and the N-protected amino acid; (b) contacting the amide conjugate with pyruvate and sialic acid aldolase under conditions appropriate to convert the amide conjugate to a sialic acid amide conjugate; (c) contacting the sialic acid amide conjugate with cytidine triphosphates, and a synthetase under conditions appropriate to form a cytidine monophosphate sialic acid amide conjugate; (d) removing the N-protecting group from the cytidine monophosphate sialic acid amide conjugate, thereby producing a free amine; and (e) contacting the free amine with an activated PEG, thereby forming the cytidine monophosphate sialic acid-poly(ethylene glycol).

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] **FIG. 1** is a general synthetic scheme of the invention for preparing an activated sialic acid-poly(ethylene glycol).

[0015] **FIG. 2** is a synthetic scheme of the invention for preparing an activated sialic acid-glycyl-poly(ethylene glycol) conjugate.

DETAILED DESCRIPTION OF THE INVENTION

Abbreviations

[0016] PEG, poly(ethyleneglycol); m-PEG, methoxy-poly(ethylene glycol); PPG, poly(propyleneglycol); m-PPG, methoxy-poly(propylene glycol); Fuc, fucosyl; Gal, galactosyl; GalNAc, N-acetylgalactosaminyl; Glc, glucosyl; GlcNAc, N-acetylglucosaminyl; Man, mannosyl; ManAc, mannosaminyl acetate; Sia, sialic acid; and NeuAc, N-acetylneuraminyl.

Definitions

[0017] Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures in cell culture, molecular genetics, organic chemistry and nucleic acid chemistry and hybridization are those well known and commonly employed in the art. The nomenclature used herein and the laboratory procedures in analytical chemistry, and organic synthetic described below are those well known and commonly employed in the art. Standard techniques, or modifications thereof, are used for chemical syntheses and chemical analyses.

[0018] The term "glycoconjugation," as used herein, refers to the enzymatically mediated conjugation of a modified sugar species to an amino acid or glycosyl residue of a peptide. A subgenus of "glycoconjugation" is "glyco-PEG-ylation," in which the modifying group of the modified sugar is poly(ethylene glycol), and alkyl derivative (e.g., m-PEG) or reactive derivative (e.g., H_2N-PEG, HOOC-PEG) thereof.

[0019] The term "sialic acid" refers to any member of a family of nine-carbon carboxylated sugars. The most common member of the sialic acid family is N-acetyl-neuraminic acid (2-keto-5-acetamido-3,5-dideoxy-D-glycero-D-galactononulopyranos-1-onic acid (often abbreviated as Neu5Ac, NeuAc, or NANA). A second member of the family is N-glycolyl-neuraminic acid (Neu5Gc or NeuGc), in which the N-acetyl group of NeuAc is hydroxylated. A third sialic acid family member is 2-keto-3-deoxy-nonulosonic acid (KDN) (Nadano *et al.* (1986) *J. Biol. Chem.* **261**: 11550-11557; Kanamori *et al.*, *J. Biol. Chem.* **265**: 21811-21819 (1990)). Also included are 9-substituted sialic acids such as a 9-O-C_1-C_6 acyl-Neu5Ac like

9-O-lactyl-Neu5Ac or 9-O-acetyl-Neu5Ac, 9-deoxy-9-fluoro-Neu5Ac and 9-azido-9-deoxy-Neu5Ac. For review of the sialic acid family, *see, e.g.*, Varki, *Glycobiology* 2: 25-40 (1992); *Sialic Acids: Chemistry, Metabolism and Function*, R. Schauer, Ed. (Springer-Verlag, New York (1992)). The synthesis and use of sialic acid compounds in a sialylation procedure is disclosed in international application WO 92/16640, published October 1, 1992.

[0020] "Peptide" refers to a polymer in which the monomers are amino acids and are joined together through amide bonds, alternatively referred to as a polypeptide. Additionally, unnatural amino acids, for example, β-alanine, phenylglycine and homoarginine are also included. Amino acids that are not gene-encoded may also be used in the present invention. Furthermore, amino acids that have been modified to include reactive groups, glycosylation sites, polymers, therapeutic moieties, biomolecules and the like may also be used in the invention. All of the amino acids used in the present invention may be either the D - or L - isomer. The L -isomer is generally preferred. In addition, other peptidomimetics are also useful in the present invention. As used herein, "peptide" refers to both glycosylated and unglycosylated peptides. Also included are peptides that are incompletely glycosylated by a system that expresses the peptide. For a general review, *see,* Spatola, A. F., in CHEMISTRY AND BIOCHEMISTRY OF AMINO ACIDS, PEPTIDES AND PROTEINS, B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983).

[0021] The term "peptide conjugate," refers to species of the invention in which a peptide is glycoconjugated with a modified sugar as set forth herein. In a representative example, the peptide is a mutant peptide having an O-linked glycosylation site not present in the wild-type peptide.

[0022] The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, *e.g.*, hydroxyproline, γ-carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, *i.e.*, an α carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, *e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical

compounds that have a structure that is different from the general chemical structure of an amino acid, but that function in a manner similar to a naturally occurring amino acid.

[0023] As used herein, the term "modified sugar," refers to a naturally- or non-naturally-occurring carbohydrate that is enzymatically added onto an amino acid or a glycosyl residue of a peptide in a process of the invention. The modified sugar is selected from a number of enzyme substrates including, but not limited to sugar nucleotides (mono-, di-, and tri-phosphates), activated sugars (*e.g.*, glycosyl halides, glycosyl mesylates) and sugars that are neither activated nor nucleotides. The "modified sugar" is covalently functionalized with a "modifying group." Useful modifying groups include, but are not limited to, water-soluble polymers, therapeutic moieties, diagnostic moieties, biomolecules and the like. The modifying group is preferably not a naturally occurring, or an unmodified carbohydrate. The locus of functionalization with the modifying group is selected such that it does not prevent the "modified sugar" from being added enzymatically to a peptide.

[0024] The term "water-soluble" refers to moieties that have some detectable degree of solubility in water. Methods to detect and/or quantify water solubility are well known in the art. Exemplary water-soluble polymers include peptides, saccharides, poly(ethers), poly(amines), poly(carboxylic acids) and the like. Peptides can have mixed sequences of be composed of a single amino acid, e.g. poly(lysine). An exemplary polysaccharide is poly(sialic acid). An exemplary poly(ether) is poly(ethylene glycol), e.g, m-PEG. Poly(ethylene imine) is an exemplary polyamine, and poly(acrylic) acid is a representative poly(carboxylic acid).

[0025] The term, "glycosyl linking group," as used herein refers to a glycosyl residue to which an agent (e.g., water-soluble polymer, therapeutic moiety, biomolecule) is covalently attached. In the methods of the invention, the "glycosyl linking group" becomes covalently attached to a glycosylated or unglycosylated peptide, thereby linking the agent to an amino acid and/or glycosyl residue on the peptide. A "glycosyl linking group" is generally derived from a "modified sugar" by the enzymatic attachment of the "modified sugar" to an amino acid and/or glycosyl residue of the peptide. An "intact glycosyl linking group" refers to a linking group that is derived from a glycosyl moiety in which the individual saccharide monomer that links the conjugate is not degraded, e.g., oxidized, e.g., by sodium metaperiodate. "Intact glycosyl linking groups" of the invention may be derived from a

naturally occurring oligosaccharide by addition of glycosyl unit(s) or removal of one or more glycosyl unit from a parent saccharide structure.

[0026] The term "targeting moiety," as used herein, refers to species that will selectively localize in a particular tissue or region of the body. The localization is mediated by specific recognition of molecular determinants, molecular size of the targeting agent or conjugate, ionic interactions, hydrophobic interactions and the like. Other mechanisms of targeting an agent to a particular tissue or region are known to those of skill in the art. Exemplary targeting moieties include antibodies, antibody fragments, transferrin, HS-glycoprotein, coagulation factors, serum proteins, β-glycoprotein, G-CSF, GM-CSF, M-CSF, EPO and the like.

[0027] As used herein, "therapeutic moiety" means any agent useful for therapy including, but not limited to, antibiotics, anti-inflammatory agents, anti-tumor drugs, cytotoxins, and radioactive agents. "Therapeutic moiety" includes prodrugs of bioactive agents, constructs in which more than one therapeutic moiety is bound to a carrier, e.g, multivalent agents. Therapeutic moiety also includes proteins and constructs that include proteins. Exemplary proteins include, but are not limited to, Erythropoietin (EPO), Granulocyte Colony Stimulating Factor (GCSF), Granulocyte Macrophage Colony Stimulating Factor (GMCSF), Interferon (e.g., Interferon-α, -β, -γ), Interleukin (e.g., Interleukin II), serum proteins (e.g., Factors VII, VIIa, VIII, IX, and X), Human Chorionic Gonadotropin (HCG), Follicle Stimulating Hormone (FSH) and Lutenizing Hormone (LH) and antibody fusion proteins (e.g. Tumor Necrosis Factor Receptor ((TNFR)/Fc domain fusion protein)).

[0028] As used herein, "anti-tumor drug" means any agent useful to combat cancer including, but not limited to, cytotoxins and agents such as antimetabolites, alkylating agents, anthracyclines, antibiotics, antimitotic agents, procarbazine, hydroxyurea, asparaginase, corticosteroids, interferons and radioactive agents. Also encompassed within the scope of the term "anti-tumor drug," are conjugates of peptides with anti-tumor activity, *e.g.* TNF-α. Conjugates include, but are not limited to those formed between a therapeutic protein and a glycoprotein of the invention. A representative conjugate is that formed between PSGL-1 and TNF-α.

[0029] As used herein, "a cytotoxin or cytotoxic agent" means any agent that is detrimental to cells. Examples include taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin,

daunorubicin, dihydroxy anthracinedione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Other toxins include, for example, ricin, CC-1065 and analogues, the duocarmycins. Still other toxins include diptheria toxin, and snake venom (e.g., cobra venom).

[0030] As used herein, "a radioactive agent" includes any radioisotope that is effective in diagnosing or destroying a tumor. Examples include, but are not limited to, indium-111, cobalt-60. Additionally, naturally occurring radioactive elements such as uranium, radium, and thorium, which typically represent mixtures of radioisotopes, are suitable examples of a radioactive agent. The metal ions are typically chelated with an organic chelating moiety.

[0031] Many useful chelating groups, crown ethers, cryptands and the like are known in the art and can be incorporated into the compounds of the invention (e.g. EDTA, DTPA, DOTA, NTA, HDTA, *etc.* and their phosphonate analogs such as DTPP, EDTP, HDTP, NTP, *etc*). *See*, for example, Pitt *et al.*, "The Design of Chelating Agents for the Treatment of Iron Overload," In, INORGANIC CHEMISTRY IN BIOLOGY AND MEDICINE; Martell, Ed.; American Chemical Society, Washington, D.C., 1980, pp. 279-312; Lindoy, THE CHEMISTRY OF MACROCYCLIC LIGAND COMPLEXES; Cambridge University Press, Cambridge,1989; Dugas, BIOORGANIC CHEMISTRY; Springer-Verlag, New York, 1989, and references contained therein.

[0032] Additionally, a manifold of routes allowing the attachment of chelating agents, crown ethers and cyclodextrins to other molecules is available to those of skill in the art. *See*, for example, Meares *et al.*, "Properties of In Vivo Chelate-Tagged Proteins and Polypeptides." In, MODIFICATION OF PROTEINS: FOOD, NUTRITIONAL, AND PHARMACOLOGICAL ASPECTS;" Feeney, *et al.*, Eds., American Chemical Society, Washington, D.C., 1982, pp. 370-387; Kasina *et al.*, *Bioconjugate Chem.*, **9**: 108-117 (1998); Song *et al.*, *Bioconjugate Chem.*, **8**: 249-255 (1997).

Modified Sugars

[0033] Modified glycosyl donor species ("modified sugars") are preferably selected from modified sugar nucleotides, activated modified sugars and modified sugars that are simple saccharides that are neither nucleotides nor activated. Any desired carbohydrate structure can be added to a peptide using the methods of the invention. Typically, the structure will be a

monosaccharide, but the present invention is not limited to the use of modified monosaccharide sugars; oligosaccharides and polysaccharides are useful as well.

[0034] The modifying group is attached to a sugar moiety by enzymatic means, chemical means or a combination thereof, thereby producing a modified sugar. The sugars are substituted at any position that allows for the attachment of the modifying moiety, yet which still allows the sugar to function as a substrate for the enzyme used to ligate the modified sugar to the peptide. In a preferred embodiment, when sialic acid is the sugar, the sialic acid is substituted with the modifying group at the 5-position on the amine moiety that is normally acetylated in sialic acid.

[0035] In certain embodiments of the present invention, the modified sugar is activated as a nucleotide. Exemplary sugar nucleotides that are used in the present invention in their modified form include nucleotide mono-, di- or triphosphates or analogs thereof. In a preferred embodiment, the modified sugar nucleotide is selected from a UDP-glycoside, CMP-glycoside, or a GDP-glycoside. Even more preferably, the modified sugar nucleotide is selected from an UDP-galactose, UDP-galactosamine, UDP-glucose, UDP-glucosamine, GDP-mannose, GDP-fucose, CMP-sialic acid, or CMP-NeuAc. N-acetylamine derivatives of the sugar nucletides are also of use in the method of the invention.

[0036] The modified sugars of the invention are conjugates between sialic acid and diverse species such as water-soluble polymers, therapeutic moieties, diagnostic moieties, targeting moieties and the like. The modified sugars are of use in forming PEG-conjugates, such as peptide conjugates, lipid conjugates and the like.

[0037] Exemplary modifying groups are discussed below. The modifying groups can be selected for their ability to impart to a peptide one or more desirable property. Exemplary properties include, but are not limited to, enhanced pharmacokinetics, enhanced pharmacodynamics, improved biodistribution, providing a polyvalent species, improved water solubility, enhanced or diminished lipophilicity, and tissue targeting.

Water-Soluble Polymers

[0038] The hydrophilicity of a selected peptide is enhanced by conjugation with polar molecules such as amine-, ester-, hydroxyl- and polyhydroxyl-containing molecules. Representative examples include, but are not limited to, polylysine, polyethyleneimine, and polyethers (e.g., poly(ethyleneglycol), alkyl-PEG (e.g., m-poly(ethylene glycol)), acyl-PEG, acyl-alkyl-PEG, alkyl-acyl-PEG carbamoyl-PEG, aryl-PEG), PPG derivatives (e.g.,

poly(propyleneglycol), alkyl-PPG (e.g., m-poly(propylene glycol)), acyl-PPG, acyl-alkyl-PPG, alkyl-acyl-PPG carbamoyl-PPG, aryl-PPG). Preferred water-soluble polymers are essentially non-fluorescent, or emit such a minimal amount of fluorescence that they are inappropriate for use as a fluorescent marker in an assay. Moreover, it is generally preferred to use polymers that are not naturally occurring sugars. An exception to this preference is the use of an otherwise naturally occurring sugar that is modified by covalent attachment of another entity (*e.g.*, poly(ethylene glycol), poly(propylene glycol), biomolecule, therapeutic moiety, diagnostic moiety, *etc.*). In another exemplary embodiment, a therapeutic sugar moiety is conjugated to a linker arm and the sugar-linker arm cassette is subsequently conjugated to a peptide via a method of the invention.

[0039] Methods and chemistry for activation of water-soluble polymers and saccharides as well as methods for conjugating saccharides and polymers to various species are described in the literature. Commonly used methods for activation of polymers include activation of functional groups with cyanogen bromide, periodate, glutaraldehyde, biepoxides, epichlorohydrin, divinylsulfone, carbodiimide, sulfonyl halides, trichlorotriazine, *etc.* (*see*, R. F. Taylor, (1991), PROTEIN IMMOBILISATION. FUNDAMENTALS AND APPLICATIONS, Marcel Dekker, N.Y.; S. S. Wong, (1992), CHEMISTRY OF PROTEIN CONJUGATION AND CROSSLINKING, CRC Press, Boca Raton; G. T. Hermanson *et al.*, (1993), IMMOBILIZED AFFINITY LIGAND TECHNIQUES, Academic Press, N.Y.; Dunn, R.L., *et al.*, Eds. POLYMERIC DRUGS AND DRUG DELIVERY SYSTEMS, ACS Symposium Series Vol. 469, American Chemical Society, Washington, D.C. 1991).

[0040] Many water-soluble polymers are known to those of skill in the art and are useful in practicing the present invention. The term water-soluble polymer encompasses species such as saccharides (e.g., dextran, amylose, hyalouronic acid, poly(sialic acid), heparans, heparins, etc.); poly (amino acids); nucleic acids; synthetic polymers (e.g., poly(acrylic acid), poly(ethers), e.g., poly(ethylene glycol); peptides, proteins, and the like. The present invention may be practiced with any water-soluble polymer with the sole limitation that the polymer must include a point at which the remainder of the conjugate can be attached.

[0041] Methods for activation of polymers can also be found in WO 94/17039, U.S. Pat. No. 5,324,844, WO 94/18247, WO 94/04193, U.S. Pat. No. 5,219,564, U.S. Pat. No. 5,122,614, WO 90/13540, U.S. Pat. No. 5,281,698, and more WO 93/15189, and for conjugation between activated polymers and peptides, *e.g.* Coagulation Factor VIII (WO 94/15625), haemoglobin (WO 94/09027), oxygen carrying molecule (U.S. Pat. No.

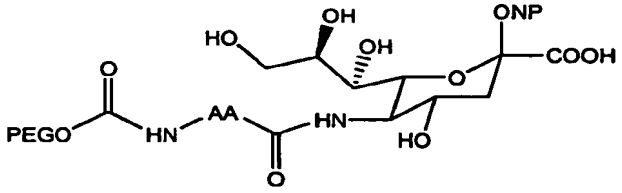
4,412,989), ribonuclease and superoxide dismutase (Veronese *at al., App. Biochem. Biotech.* **11**: 141-45 (1985)).

[0042] Preferred water-soluble polymers are those in which a substantial proportion of the polymer molecules in a sample of the polymer are of approximately the same molecular weight; such polymers are "homodisperse."

[0043] The present invention is further illustrated by reference to a poly(ethylene glycol) or monomethoxy-poly(ethylene glycol) (m-PEG) conjugate. Several reviews and monographs on the functionalization and conjugation of PEG are available. *See,* for example, Harris, *Macronol. Chem. Phys.* **C25**: 325-373 (1985); Scouten, *Methods in Enzymology* **135**: 30-65 (1987); Wong *et al., Enzyme Microb. Technol.* **14**: 866-874 (1992); Delgado *et al., Critical Reviews in Therapeutic Drug Carrier Systems* **9**: 249-304 (1992); Zalipsky, *Bioconjugate Chem.* **6**: 150-165 (1995); and Bhadra, *et al., Pharmazie,* **57**:5-29 (2002).

[0044] The poly(ethylene glycol) useful in forming the conjugate of the invention is either linear or branched.

[0045] In selected glyco-PEG-ylated peptides of the invention, the PEG-intact glycosyl linker cassette has the structure:



in which AA is an amino acid residue, PEG is poly(ethylene glycol) or methoxy-poly(ethylene glycol) and NP is a nucleotide, which is linked to the glycosyl moiety via a phosphodiester bond ("nucleotide phosphate").

[0046] PEG moieties of any molecular weight, e.g., 5 Kd, 10 Kd, 20 Kd and 30kD are of use in the present invention. In a preferred embodiment, the PEG molecular weights are number-average molecular weights, which correlate closely to the peak-average molecular weight.

Biomolecules

[0047] In another preferred embodiment, the modified sugar bears a biomolecule. In still further preferred embodiments, the biomolecule is a functional protein, enzyme, antigen, antibody, peptide, nucleic acid (*e.g.*, single nucleotides or nucleosides, oligonucleotides,

polynucleotides and single- and higher-stranded nucleic acids), lectin, receptor or a combination thereof.

[0048] Preferred biomolecules are essentially non-fluorescent, or emit such a minimal amount of fluorescence that they are inappropriate for use as a fluorescent marker in an assay. Moreover, it is generally preferred to use biomolecules that are not sugars. An exception to this preference is the use of an otherwise naturally occurring sugar that is modified by covalent attachment of another entity (*e.g.*, PEG, biomolecule, therapeutic moiety, diagnostic moiety, *etc.*). In an exemplary embodiment, a sugar moiety, which is a biomolecule, is conjugated to a linker arm and the sugar-linker arm cassette is subsequently conjugated to a peptide via a method of the invention.

[0049] Biomolecules useful in practicing the present invention can be derived from any source. The biomolecules can be isolated from natural sources or they can be produced by synthetic methods. Peptides can be natural peptides or mutated peptides. Mutations can be effected by chemical mutagenesis, site-directed mutagenesis or other means of inducing mutations known to those of skill in the art. Peptides useful in practicing the instant invention include, for example, enzymes, antigens, antibodies and receptors. Antibodies can be either polyclonal or monoclonal; either intact or fragments. The peptides are optionally the products of a program of directed evolution

[0050] Both naturally derived and synthetic peptides and nucleic acids are of use in conjunction with the present invention; these molecules can be attached to a sugar residue component or a crosslinking agent by any available reactive group. For example, peptides can be attached through a reactive amine, carboxyl, sulfhydryl, or hydroxyl group. The reactive group can reside at a peptide terminus or at a site internal to the peptide chain. Nucleic acids can be attached through a reactive group on a base (*e.g.*, exocyclic amine) or an available hydroxyl group on a sugar moiety (*e.g.*, 3'- or 5'-hydroxyl). The peptide and nucleic acid chains can be further derivatized at one or more sites to allow for the attachment of appropriate reactive groups onto the chain. *See,* Chrisey *et al. Nucleic Acids Res.* **24:** 3031-3039 (1996).

[0051] In a further preferred embodiment, the biomolecule is selected to direct the peptide modified by the methods of the invention to a specific tissue, thereby enhancing the delivery of the peptide to that tissue relative to the amount of underivatized peptide that is delivered to the tissue. In a still further preferred embodiment, the amount of derivatized peptide

delivered to a specific tissue within a selected time period is enhanced by derivatization by at least about 20%, more preferably, at least about 40%, and more preferably still, at least about 100%. Presently, preferred biomolecules for targeting applications include antibodies, hormones and ligands for cell-surface receptors.

[0052] In still a further exemplary embodiment, there is provided as conjugate with biotin. Thus, for example, a selectively biotinylated peptide is elaborated by the attachment of an avidin or streptavidin moiety bearing one or more modifying groups.

Therapeutic Moieties

[0053] In another preferred embodiment, the modified sugar includes a therapeutic moiety. Those of skill in the art will appreciate that there is overlap between the category of therapeutic moieties and biomolecules; many biomolecules have therapeutic properties or potential.

[0054] The therapeutic moieties can be agents already accepted for clinical use or they can be drugs whose use is experimental, or whose activity or mechanism of action is under investigation. The therapeutic moieties can have a proven action in a given disease state or can be only hypothesized to show desirable action in a given disease state. In a preferred embodiment, the therapeutic moieties are compounds, which are being screened for their ability to interact with a tissue of choice. Therapeutic moieties, which are useful in practicing the instant invention include drugs from a broad range of drug classes having a variety of pharmacological activities. Preferred therapeutic moieties are essentially non-fluorescent, or emit such a minimal amount of fluorescence that they are inappropriate for use as a fluorescent marker in an assay. Moreover, it is generally preferred to use therapeutic moieties that are not sugars. An exception to this preference is the use of a sugar that is modified by covalent attachment of another entity, such as a PEG, biomolecule, therapeutic moiety, diagnostic moiety and the like. In another exemplary embodiment, a therapeutic sugar moiety is conjugated to a linker arm and the sugar-linker arm cassette is subsequently conjugated to a peptide via a method of the invention.

[0055] Methods of conjugating therapeutic and diagnostic agents to various other species are well known to those of skill in the art. *See,* for example Hermanson, BIOCONJUGATE TECHNIQUES, Academic Press, San Diego, 1996; and Dunn *et al.*, Eds. POLYMERIC DRUGS AND DRUG DELIVERY SYSTEMS, ACS Symposium Series Vol. 469, American Chemical Society, Washington, D.C. 1991.

[0056] In an exemplary embodiment, the therapeutic moiety is attached to the modified sugar via a linkage that is cleaved under selected conditions. Exemplary conditions include, but are not limited to, a selected pH (e.g., stomach, intestine, endocytotic vacuole), the presence of an active enzyme (e.g, esterase, reductase, oxidase), light, heat and the like. Many cleavable groups are known in the art. *See*, for example, Jung *et al.*, *Biochem. Biophys. Acta*, **761**: 152-162 (1983); Joshi *et al.*, *J. Biol. Chem.*, **265**: 14518-14525 (1990); Zarling *et al.*, *J. Immunol.*, **124**: 913-920 (1980); Bouizar *et al.*, *Eur. J. Biochem.*, **155**: 141-147 (1986); Park *et al.*, *J. Biol. Chem.*, **261**: 205-210 (1986); Browning *et al.*, *J. Immunol.*, **143**: 1859-1867 (1989).

Preparation of Modified Sugars

[0057] In general, the sugar moiety and the modifying group are linked together through the use of reactive groups, which are typically transformed by the linking process into a new organic functional group or unreactive species. The sugar reactive functional group(s), is located at any position on the sugar moiety. Reactive groups and classes of reactions useful in practicing the present invention are generally those that are well known in the art of bioconjugate chemistry. Currently favored classes of reactions available with reactive sugar moieties are those, which proceed under relatively mild conditions. These include, but are not limited to nucleophilic substitutions (*e.g.*, reactions of amines and alcohols with acyl halides, active esters), electrophilic substitutions (*e.g.*, enamine reactions) and additions to carbon-carbon and carbon-heteroatom multiple bonds (*e.g.*, Michael reaction, Diels-Alder addition). These and other useful reactions are discussed in, for example, March, ADVANCED ORGANIC CHEMISTRY, 3rd Ed., John Wiley & Sons, New York, 1985; Hermanson, BIOCONJUGATE TECHNIQUES, Academic Press, San Diego, 1996; and Feeney *et al.*, MODIFICATION OF PROTEINS; Advances in Chemistry Series, Vol. 198, American Chemical Society, Washington, D.C., 1982.

[0058] Useful reactive functional groups pendent from a sugar nucleus or modifying group include, but are not limited to:

(a) carboxyl groups and various derivatives thereof including, but not limited to, N-hydroxysuccinimide esters, N-hydroxybenztriazole esters, acid halides, acyl imidazoles, thioesters, p-nitrophenyl esters, alkyl, alkenyl, alkynyl and aromatic esters;

(b) hydroxyl groups, which can be converted to, *e.g.*, esters, ethers, aldehydes, *etc.*

(c) haloalkyl groups, wherein the halide can be later displaced with a nucleophilic group such as, for example, an amine, a carboxylate anion, thiol anion, carbanion, or an alkoxide ion, thereby resulting in the covalent attachment of a new group at the functional group of the halogen atom;

(d) dienophile groups, which are capable of participating in Diels-Alder reactions such as, for example, maleimido groups;

(e) aldehyde or ketone groups, such that subsequent derivatization is possible via formation of carbonyl derivatives such as, for example, imines, hydrazones, semicarbazones or oximes, or via such mechanisms as Grignard addition or alkyllithium addition;

(f) sulfonyl halide groups for subsequent reaction with amines, for example, to form sulfonamides;

(g) thiol groups, which can be, for example, converted to disulfides or reacted with acyl halides;

(h) amine or sulfhydryl groups, which can be, for example, acylated, alkylated or oxidized;

(i) alkenes, which can undergo, for example, cycloadditions, acylation, Michael addition, *etc*; and

(j) epoxides, which can react with, for example, amines and hydroxyl compounds.

[0059] The reactive functional groups can be chosen such that they do not participate in, or interfere with, the reactions necessary to assemble the reactive sugar nucleus or modifying group. Alternatively, a reactive functional group can be protected from participating in the reaction by the presence of a protecting group. Those of skill in the art understand how to protect a particular functional group such that it does not interfere with a chosen set of reaction conditions. For examples of useful protecting groups, *see,* for example, Greene *et al.*, PROTECTIVE GROUPS IN ORGANIC SYNTHESIS, John Wiley & Sons, New York, 1991.

[0060] In the discussion that follows, a number of specific examples of modified sugars that are useful in practicing the present invention are set forth. In the exemplary embodiments, a sialic acid derivative is utilized as the sugar nucleus to which the modifying group is attached. The focus of the discussion on sialic acid derivatives is for clarity of illustration only and should not be construed to limit the scope of the invention. Those of

skill in the art will appreciate that a variety of other sugar moieties can be activated and derivatized in a manner analogous to that set forth using sialic acid as an example. For example, numerous methods are available for modifying galactose, glucose, N-acetylgalactosamine and fucose to name a few sugar substrates, which are readily modified by art recognized methods. *See*, for example, Elhalabi *et al.*, *Curr. Med. Chem.* **6**: 93 (1999); and Schafer *et al.*, *J. Org. Chem.* **65**: 24 (2000)).

[0061] In an exemplary embodiment, the peptide that is modified by a method of the invention is a glycopeptide that is produced in mammalian cells (e.g., CHO cells) or in a transgenic animal and thus contains N- and/or O-linked oligosaccharide chains, which are incompletely sialylated. The oligosaccharide chains of the glycopeptide lacking a sialic acid and containing a terminal galactose residue can be glyco-PEG-ylated, glyco-PPG-ylated or otherwise modified with a modified sialic acid.

[0062] In **FIG. 1**, a general scheme according to the present invention is set forth. Thus, according to **FIG. 1**, an amide conjugate between mannosamine and a protected amino acid is formed by contacting mannosamine with an N-protected amino acid under conditions appropriate to form the conjugate. The carboxyl terminus of the protected amino acid is activated *in situ* or it is optionally converted to a reactive group that is stable to storage, e.g., N-hydroxy-succinimide. The amino acid can be selected from any natural or non-natural amino acid. Those of skill in the art understand how to protect side-chain amino acids from undesirably reacting in the method of the invention. The amide conjugate is reacted with pyruvate and sialic acid aldolase under conditions appropriate to convert the amide conjugate to a sialic acid amide conjugate, which is subsequently converted to a nucleotide phosphate sialic acid amide conjugate by reaction of the sialic acid amide conjugate with a precursor of the nucleotide phosphate and an appropriate enzyme. In an exemplary embodiment, the precursor is cytidine triphosphate and the enzyme is a synthetase. Following the formation of the nucleotide sugar, the amino acid amine is deprotected, providing a free, reactive amine amine. The amine serves as a locus for conjugating the modifying moiety to the nucleotide sugar. In **FIG. 1**, the modifying moiety is exemplified by a water-soluble polymer, i.e., poly(ethylene glycol), e.g., PEG, m-PEG, etc.

The present invention is further exemplified in **FIG. 2**, which sets forth a scheme for preparing sialic acid-glycyl-PEG-cytidine monophosphate. Similar to the scheme set forth in **FIG. 1**, that of **FIG. 2** originates with mannosamine. The sugar is conjugated with FMOC-glycine, using the N-hydroxysuccinimide activated derivative of the protected amino acid.

The resulting amide conjugate is converted to the corresponding sialic acid by the action of sialic acid aldolase on the conjugate and pyruvate. The resulting sialic acid conjugate is converted to the cytidine monophosphate analogue using cytidine triphosphate and a synthetase. The CMP-analogue is deprotected by removing the protecting group from the amino acid amine moiety, converting this moiety to a reactive locus for conjugation. The amine moiety is reacted with an activated PEG species (m-PEG-O-nitrophenyl carbonate), thereby forming the sialic acid-glycyl-PEG-cytidine monophosphate.

Peptide Conjugates

[0063] In an exemplary embodiment, peptide conjugates are formed by the enzymatic attachment of a modified sugar of the invention to a glycosylated or unglycosylated peptide. The modified sugar, when interposed between the peptide (or glycosyl residue) and the modifying group on the sugar becomes what is referred to herein as "an intact glycosyl linking group." Using the exquisite selectivity of enzymes, such as glycosyltransferases, the present method provides peptides that bear a desired group at one or more specific locations. Thus, according to the present invention, a modified sugar is attached directly to a selected locus on the peptide chain or, alternatively, the modified sugar is appended onto a carbohydrate moiety of a glycopeptide. Peptides in which modified sugars are bound to both a glycopeptide carbohydrate and directly to an amino acid residue of the peptide backbone are also within the scope of the present invention.

[0064] In an exemplary embodiment, the conjugate formed using a modified sugar of the invention includes a water-soluble polymer. The *in vivo* half-life of therapeutic glycopeptides can be enhanced with water-soluble polymers such as polyethylene glycol (PEG, m-PEG) and polypropylene glycol (PPG). For example, chemical modification of proteins with PEG (PEG-ylation, m-PEG-ylation) increases their molecular size and decreases their surface- and functional group-accessibility, each of which are dependent on the size of the PEG attached to the protein. This results in an improvement of plasma half-lives and in proteolytic-stability, and a decrease in immunogenicity and hepatic uptake (Chaffee *et al. J. Clin. Invest.* **89**: 1643-1651 (1992); Pyatak *et al. Res. Commun. Chem. Pathol Pharmacol.* **29**: 113-127 (1980)). PEGylation of interleukin-2 has been reported to increase its antitumor potency *in vivo* (Katre *et al. Proc. Natl. Acad. Sci. USA.* **84**: 1487-1491 (1987)) and PEG-ylation of a F(ab')2 derived from the monoclonal antibody A7 has improved its tumor localization (Kitamura *et al. Biochem. Biophys. Res. Commun.* **28**: 1387-

1394 (1990)). Thus, in another preferred embodiment, the *in vivo* half-life of a peptide derivatized with a water-soluble polymer by a method of the invention is increased relevant to the *in vivo* half-life of the non-derivatized peptide.

[0065] The increase in peptide *in vivo* half-life is best expressed as a range of percent increase in this quantity. The lower end of the range of percent increase is about 40%, about 60%, about 80%, about 100%, about 150% or about 200%. The upper end of the range is about 60%, about 80%, about 100%, about 150%, or more than about 250%.

Sialyltransferases

[0066] The modified sialic acid species of the invention preferably serve as substrates for at least one sialyltransferase. The discussion below is generally relevant to sialyltransferases for which the modified sugars of the invention are substrates. The focus of the discussion is for clarity of illustration and should not be interpreted as limiting the sialyltransferases with which the present invention can be practiced.

[0067] Sialyltransferases are another type of glycosyltransferase that is useful in the recombinant cells and reaction mixtures of the invention. Cells that produce recombinant sialyltransferases will also produce CMP-sialic acid, which is a sialic acid donor for sialyltransferases. Examples of sialyltransferases that are suitable for use in the present invention include ST3Gal III (*e.g.*, a rat or human ST3Gal III), ST3Gal IV, ST3Gal I, ST6Gal I, ST3Gal V, ST6Gal II, ST6GalNAc I, ST6GalNAc II, and ST6GalNAc III (the sialyltransferase nomenclature used herein is as described in Tsuji *et al., Glycobiology* 6: v-xiv (1996)). An exemplary α(2,3)sialyltransferase referred to as α(2,3)sialyltransferase (EC 2.4.99.6) transfers sialic acid to the non-reducing terminal Gal of a Galβ1→3Glc disaccharide or glycoside. *See,* Van den Eijnden *et al., J. Biol. Chem.* **256:** 3159 (1981), Weinstein *et al., J. Biol. Chem.* **257:** 13845 (1982) and Wen *et al., J. Biol. Chem.* **267:** 21011 (1992). Another exemplary α2,3-sialyltransferase (EC 2.4.99.4) transfers sialic acid to the non-reducing terminal Gal of the disaccharide or glycoside. *see,* Rearick *et al., J. Biol. Chem.* **254:** 4444 (1979) and Gillespie *et al., J. Biol. Chem.* **267:** 21004 (1992). Further exemplary enzymes include Gal-β-1,4-GlcNAc α-2,6 sialyltransferase (*See*, Kurosawa *et al. Eur. J. Biochem.* **219:** 375-381 (1994)).

[0068] Preferably, for glycosylation of carbohydrates of glycopeptides the sialyltransferase will be able to transfer sialic acid to the sequence Galβ1,4GlcNAc-, the most common

penultimate sequence underlying the terminal sialic acid on fully sialylated carbohydrate structures (*see,* Table 1).

Table 1: Sialyltransferases which use the Galβ1,4GlcNAc sequence as an acceptor substrate

Sialyltransferase	Source	Sequence(s) formed	Ref.
ST6Gal I	Mammalian	NeuAcI2,6Galβ1,4GlCNAc-	1
ST3Gal III	Mammalian	NeuAcI2,3Galβ1,4GlCNAc- NeuAcI2,3Galβ1,3GlCNAc-	1
ST3Gal IV	Mammalian	NeuAcI2,3Galβ1,4GlCNAc- NeuAcI2,3Galβ1,3GlCNAc-	1
ST6Gal II	Mammalian	NeuAcI2,6Galβ1,4GlCNA	
ST6Gal II	photobacterium	NeuAcI2,6Galβ1,4GlCNAc-	2
ST3Gal V	*N. meningitides* *N. gonorrhoeae*	NeuAcI2,3Galβ1,4GlCNAc-	3

1) Goochee *et al., Bio/Technology* **9**: 1347-1355 (1991)
2) Yamamoto *et al., J. Biochem.* **120**: 104-110 (1996)
3) Gilbert *et al., J. Biol. Chem.* **271**: 28271-28276 (1996)

[0069] An example of a sialyltransferase that is useful in the claimed methods is ST3Gal III, which is also referred to as α(2,3)sialyltransferase (EC 2.4.99.6). This enzyme catalyzes the transfer of sialic acid to the Gal of a Galβ1,3GlcNAc or Galβ1,4GlcNAc glycoside (*see, e.g.*, Wen *et al., J. Biol. Chem.* **267**: 21011 (1992); Van den Eijnden *et al., J. Biol. Chem.* **256:** 3159 (1991)) and is responsible for sialylation of asparagine-linked oligosaccharides in glycopeptides. The sialic acid is linked to a Gal with the formation of an α-linkage between the two saccharides. Bonding (linkage) between the saccharides is between the 2-position of NeuAc and the 3-position of Gal. This particular enzyme can be isolated from rat liver (Weinstein *et al., J. Biol. Chem.* **257**: 13845 (1982)); the human cDNA (Sasaki *et al.* (1993) *J. Biol. Chem.* **268**: 22782-22787; Kitagawa & Paulson (1994) *J. Biol. Chem.* **269**: 1394-1401) and genomic (Kitagawa *et al.* (1996) *J. Biol. Chem.* **271**: 931-938) DNA sequences are known, facilitating production of this enzyme by recombinant expression. In a preferred embodiment, the claimed sialylation methods use a rat ST3Gal III.

[0070] Other exemplary sialyltransferases of use in the present invention include those isolated from Campylobacter jejuni, including the α(2,3). *See*, e.g, WO99/49051.

[0071] Sialyltransferases other those listed in Table 5, are also useful in an economic and efficient large-scale process for sialylation of commercially important glycopeptides. As a

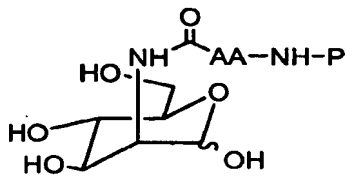
simple test to find out the utility of these other enzymes, various amounts of each enzyme (1-100 mU/mg protein) are reacted with asialo-α_1 AGP (at 1-10 mg/ml) to compare the ability of the sialyltransferase of interest to sialylate glycopeptides relative to either bovine ST6Gal I, ST3Gal III or both sialyltransferases. Alternatively, other glycopeptides or glycopeptides, or N-linked oligosaccharides enzymatically released from the peptide backbone can be used in place of asialo-α_1 AGP for this evaluation. Sialyltransferases with the ability to sialylate N-linked oligosaccharides of glycopeptides more efficiently than ST6Gal I are useful in a practical large-scale process for peptide sialylation.

[0072] While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention.

[0073] All patents, patent applications, and other publications cited in this application are incorporated by reference in the entirety.

WHAT IS CLAIMED IS:

1. A compound having the formula:



wherein

AA-NH is an amino acid residue; and

P is a protecting group.

2. The compound according to claim 1, wherein -AA-NH is $-CH_2NH$.

3. The compound according to claim 1, wherein P is FMOC.

4. A method of preparing cytidine monophosphate sialic acid-poly(ethylene glycol), said method comprising:

(a) contacting mannosamine with an activated, N-protected amino acid under conditions appropriate to form an amide conjugate between said mannosamine and the N-protected amino acid;

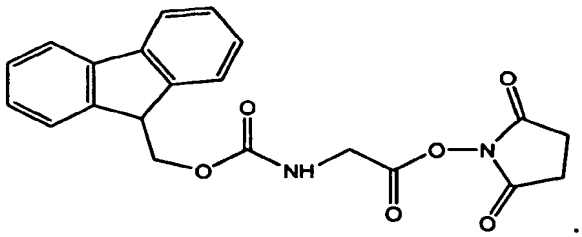
(b) contacting said amide conjugate with pyruvate and sialic acid aldolase under conditions appropriate to convert said amide conjugate to a sialic acid amide conjugate;

(c) contacting said sialic acid amide conjugate with cytidine triphosphates, and a synthetase under conditions appropriate to form a cytidine monophosphate sialic acid amide conjugate;

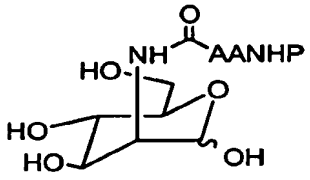
(d) removing the N-protecting group from said cytidine monophosphate sialic acid amide conjugate, thereby producing a free amine; and

(e) contacting said free amine with an activated PEG, thereby forming said cytidine monophosphate sialic acid-poly(ethylene glycol).

5. The method according to claim 4, wherein said activated N-protected amino acid has the formula:



6. The method according to claim 4, wherein said amide conjugate between said mannosamine and the N-protected amino acid has the formula:

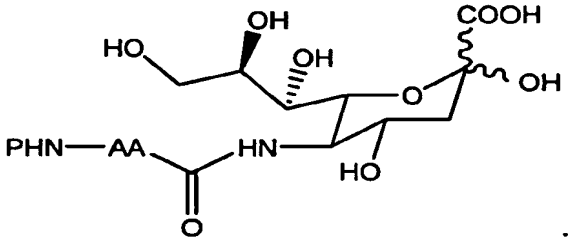


wherein

AANH is an amino acid residue; and

P is a protecting group.

7. The method according to claim 4, wherein said sialic acid amide conjugate has the formula:



PEG-YLATED NUCLEOTIDE SUGARS

ABSTRACT OF THE DISCLOSURE

The present invention provides a method of preparing a PEG-derivatized, activated sialic acid. The PEG-derivatized sialic acid is activated as a nucleotide sugar and is a substrate for a sialyltransferase, allowing the facile conjugation of a PEG-sialic acid moiety to another entity, e.g, peptide, glycopeptide, lipid, glycolipid, etc.

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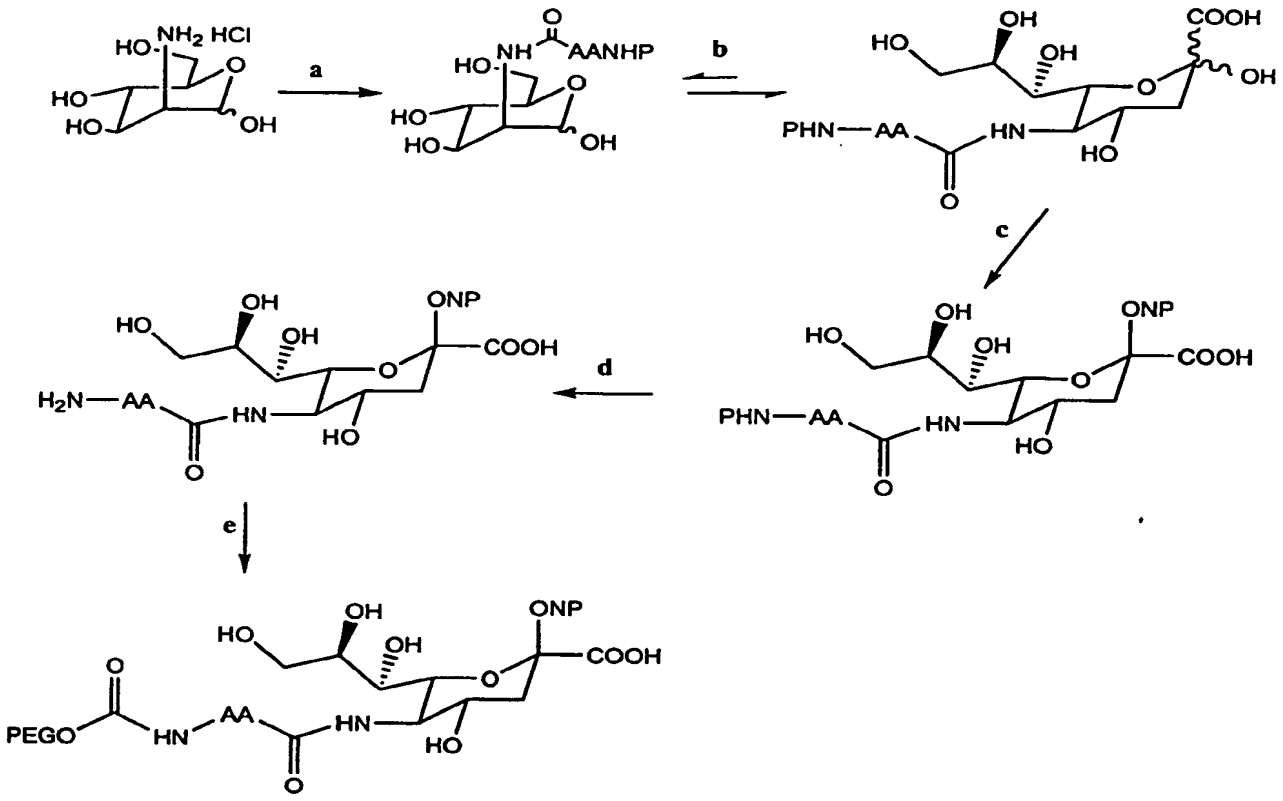
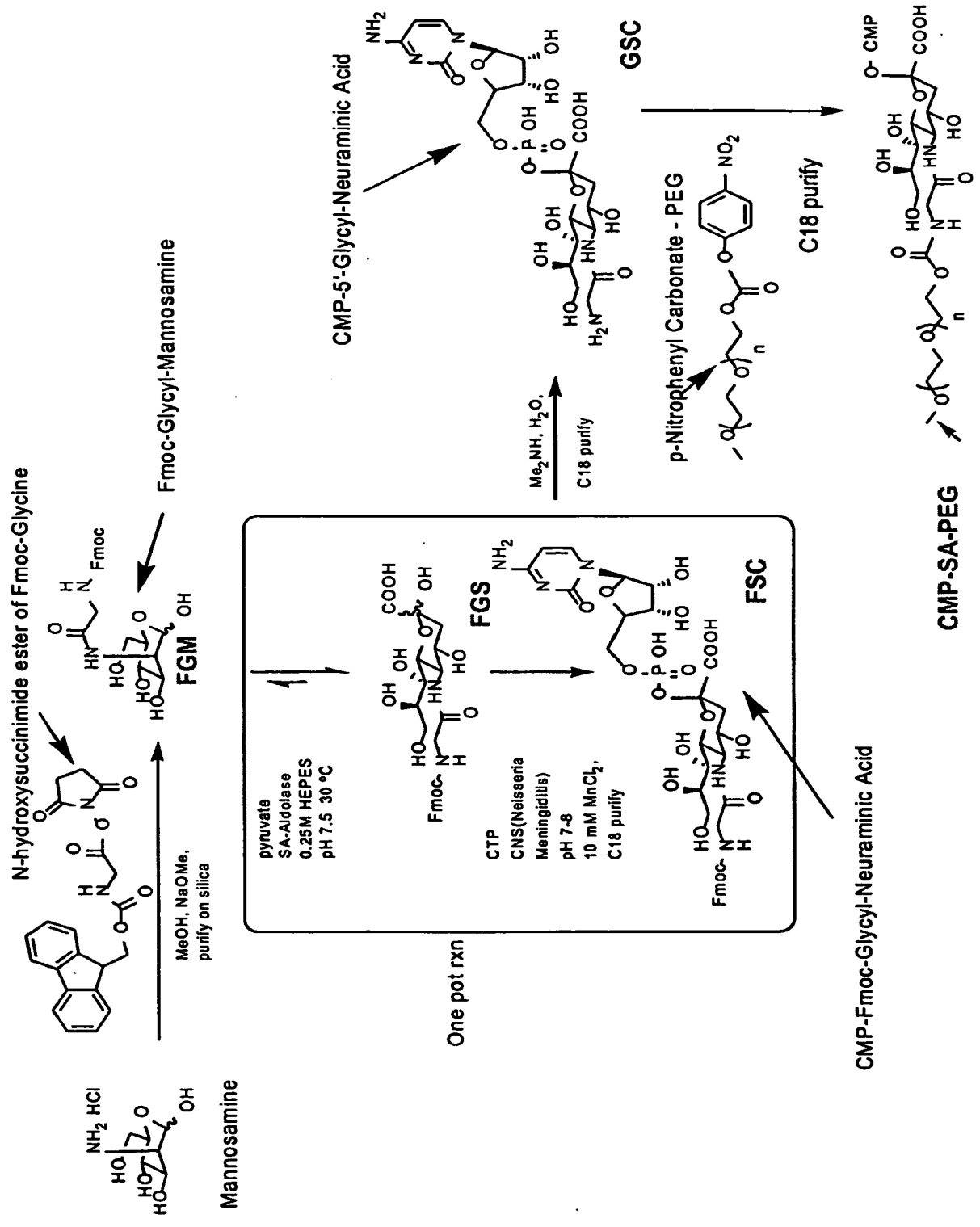
FIG. 1



a. $AG^1—O$ (O) AANHP , base; b. pyruvate, AS-Aldolase, buffer; c. synthetase, nucelotide phosphate; d. deprotecting reagent; e. activated PEG

NP = nucleotide phosphate; P = protecting group; PEG = poly(ethylene glycol) or methoxy-poly(ethylene glycol)

FIG. 2


Mannosamine
N-hydroxysuccinimide ester of Fmoc-Glycine
MeOH, NaOMe,
purify on silica
Fmoc-Glycyl-Mannosamine
FGM
One pot rxn
pyruvate
SA-Aldolase
0.25M HEPES
pH 7.5 30 °C
FGS
CTP
CNS(Neisseria
Meningidilis)
pH 7-8
10 mM MnCl2,
C18 purify
FSC
CMP-Fmoc-Glycyl-Neuraminic Acid
Me2NH, H2O,
C18 purify
CMP-5'-Glycyl-Neuraminic Acid
GSC
p-Nitrophenyl Carbonate - PEG
C18 purify
CMP-SA-PEG

Application Data Sheet

Application Information

Application number::	
Filing Date::	01/26/04
Application Type::	Provisional
Subject Matter::	Utility
Suggested classification::	
Suggested Group Art Unit::	
CD-ROM or CD-R??::	
Number of CD disks::	
Number of copies of CDs::	
Sequence Submission::	
Computer Readable Form (CRF)?::	
Number of copies of CRF::	
Title::	PEG-ylated Nucleotide Sugars
Attorney Docket Number::	019957-019600US
Request for Early Publication::	No
Request for Non-Publication::	No
Suggested Drawing Figure::	
Total Drawing Sheets::	2
Small Entity?::	Yes
Latin name::	
Variety denomination name::	
Petition included?::	No
Petition Type::	
Licensed US Govt. Agency::	
Contract or Grant Numbers One::	
Secrecy Order in Parent Appl.::	No

Applicant Information

Applicant Authority Type::	Inventor
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Name Suffix::	
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State or Province of mailing address::	PA
Country of mailing address::	US
Postal or Zip Code of mailing address::	19454

Applicant Authority Type::	Inventor
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Status::	Full Capacity
Given Name::	Caryn
Middle Name::	
Family Name::	Bowe
Name Suffix::	
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State or Province of Residence::	PA
Country of Residence::	US
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State or Province of mailing address::	PA

Country of mailing address:: US

Postal or Zip Code of mailing address:: 19044

Correspondence Information

Correspondence Customer Number:: 20350

Representative Information

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